



CD4-binding site alterations in CCR5-using HIV-1 envelopes influencing gp120–CD4 interactions and fusogenicity

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ABSTRACT

CD4-binding site (CD4bs) alterations in gp120 contribute to different pathophysiological phenotypes of CCR5-using (R5) HIV-1 strains, but the potential structural basis is unknown. Here, we characterized functionally diverse R5 envelope (Env) clones ($n=16$) to elucidate potential structural alterations within the gp120 CD4bs that influence Env function. Initially, we showed that the magnitude of gp120–CD4-binding correlates with increased fusogenicity and reduced CD4 dependence. Analysis of three-dimensional gp120 structural models revealed two CD4bs variants, D279 and N362, that were associated with reduced CD4 dependence. Further structural analysis showed that a wider aperture of the predicted CD4bs cavity, as constrained by the inner-most atoms at the gp120 V1V2 stem and the V5 loop, was associated with amino acid alterations within V5 and correlated with increased gp120–CD4 binding and increased fusogenicity. Our results provide evidence that the gp120 V5 loop may alter CD4bs conformation and contribute to increased gp120–CD4 interactions and Env fusogenicity.

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Introduction

The trimeric envelope glycoprotein (Env) of human immunodeficiency virus type 1 (HIV-1) mediates virus entry into cells, which comprises surface gp120 glycoproteins non-covalently linked to transmembrane gp41 glycoproteins that embed the complex into the viral membrane (Chan et al., 1997; Kwong et al., 1998, 2000). HIV-1 entry is initiated by gp120 binding to cellular CD4, which is a high-affinity interaction that facilitates the initial attachment of virus to the target cell (Dalglish et al., 1984). The binding of gp120 to CD4 results in dramatic conformational changes in gp120 that expose the binding site for a secondary coreceptor, which is either of the chemokine receptors CCR5 or CXCR4 [reviewed in Doms, 2000; Doms and Trono, 2000].

Crystallographic and biochemical studies of gp120 have provided valuable insights into mechanisms involved in CD4-binding and CD4-

induced conformational changes that result in formation and exposure of the coreceptor binding site (Chen et al., 2005b; Huang et al., 2005; Kwong et al., 1998; Myszka et al., 2000). The unliganded gp120 core of the closely related simian immunodeficiency virus (SIV) consists of a highly conserved inner domain, which faces the trimer axis, and a heavily glycosylated, globular outer domain, which is mostly exposed on the surface of the trimer (Chen et al., 2005b; Kwong et al., 1998; Wyatt et al., 1998; Wyatt and Sodroski, 1998). CD4 interacts with gp120 via surface-exposed residues within three separate regions distributed over six segments of gp120. These regions include the α -helices of the inner domain, the CD4-binding loop of outer domain, and the β 20– β 21 ribbon, which becomes part of the gp120 bridging sheet, which is a structural element of gp120 formed after CD4 binding that is involved in coreceptor binding (Kwong et al., 1998; Wyatt et al., 1998). Thermodynamic and structural analysis of the gp120–CD4 interaction demonstrated little evidence of a structured CD4-binding pocket on the unliganded gp120, and that CD4bs elements that influence gp120–CD4 affinity are formed from conformational alterations that occur after gp120 has encountered CD4 (Kwong et al., 2000; Myszka et al., 2000).

CD4 binding to gp120 not only drives the folding of the bridging sheet but also draws the bridging sheet, inner domain, and outer

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domains of gp120 into closer proximity to form the coreceptor binding site (Chen et al., 2005a). The burial of extensive surface at the CD4–gp120 interface provides the energy required to drive these structural rearrangements, permitting the exposure of gp120 residues involved in coreceptor binding (Myszka et al., 2000). Current models of CD4-bound gp120 binding to coreceptor suggest the gp120 V3 loop interacts principally with the coreceptor second extracellular loop (ECL2) region, while the gp120 bridging sheet interacts with the coreceptor N-terminus (Brelot et al., 1999; Cormier and Dragic, 2002; Farzan et al., 1999; Huang et al., 2005). Mutagenesis studies demonstrated a critical role for tyrosine sulfation of the coreceptor N-terminus, particularly at amino acid positions 14 and 15 of CCR5, in facilitating HIV-1 entry (Cormier et al., 2000; Farzan et al., 1999). The ECL1 and ECL3 regions may also influence coreceptor function of CCR5 and CXCR4 (Doranz et al., 1997, 1999; Farzan et al., 1998). The interaction of CD4-bound gp120 with coreceptor induces additional conformational changes in gp120, which leads to a structural rearrangement in gp41 that enables fusion and virus entry.

In addition to mediating HIV-1 entry into cells, the Env glycoproteins contribute to viral pathogenicity through fusogenic properties. Env mediates most of the acute cytopathic effects of HIV-1 infection in cultured cells (Sodroski et al., 1986), and membrane fusion appears to be an important factor contributing to HIV-1 cytopathicity *in vitro* (LaBonte et al., 2000; Wade et al., 2010). Passage of chimeric simian-HIV (SHIV) strains in macaques demonstrated enhancement of pathogenicity that often resulted from increased Env-mediated membrane fusing capacity (Etemad-Moghadam et al., 2001; Etemad-Moghadam et al., 2000; Karlsson et al., 1998; Liu et al., 1999; Si et al., 2004), suggesting that fusogenicity contributes to viral pathogenicity in this animal model. In addition, the cytopathic effects of Env-mediated HIV-1 fusogenicity are evident in humans. For example, the presence of multinucleated giant cells (MNGC) in brain, formed by Env-mediated fusion between infected and uninfected macrophage lineage cells, is characteristic of HIV-1 encephalitis (HIVE) and a neuropathological hallmark of HIV-associated dementia (Gonzalez-Scarano and Martin-Garcia, 2005).

The Env determines cellular tropism of HIV-1 through alterations in gp120 that influence fusogenicity. The ability of CCR5-using (R5) HIV-1 strains to enter cell types that have relatively low levels of cell-surface CD4 expression, for example, macrophages and microglia, is frequently attributed to augmented binding of gp120 to CD4 and/or greater exposure of the CD4-binding site (CD4bs) in gp120, which enables HIV-1 to scavenge limiting levels of CD4 to mediate virus–cell fusion (Duenas-Decamp et al., 2009; Dunfee et al., 2006, 2007, 2009; Gorry et al., 2001, 2002; Martin et al., 2001; Martin-Garcia et al., 2005; Peters et al., 2006, 2008; Rossi et al., 2008; Thomas et al., 2007). Furthermore, enhanced Env-mediated fusogenicity is a prominent phenotype of R5 HIV-1 strains isolated from subjects with AIDS (Sterjovski et al., 2007) and causes increased levels of Env-mediated apoptosis in CD4+ cells (Wade et al., 2010). Thus, an enhanced interaction between gp120 and CD4 influences Env-mediated fusogenicity and viral pathogenicity. However, the structural alterations occurring within the CD4bs of primary gp120 proteins that contribute to augmented gp120–CD4 interactions and subsequent cytopathicity are presently unclear.

In this study, we characterized a panel of functionally diverse R5 Env clones to better understand potential structural alterations within the gp120 CD4bs, which may influence CD4 interactions and fusogenicity.

Results

CD4 binding influences Env fusogenicity and the ability to use low levels of CD4

Enhanced fusogenicity is a prominent phenotype of R5 Envs that have increased cytopathicity for CD4+ T cells (Sterjovski et al., 2007;

Wade et al., 2010) and of SHIV variants passed in macaques that acquired enhanced pathogenicity *in vivo* (Etemad-Moghadam et al., 2001; Etemad-Moghadam et al., 2000; Karlsson et al., 1998; Liu et al., 1999; Si et al., 2004). To determine whether enhanced fusogenicity of R5 Envs is associated with ability to bind CD4, gp120–CD4-binding assays were conducted using a panel of primary isolate-derived R5 Envs that exhibit diversity in levels of cell–cell fusion activity (Sterjovski et al., 2007) (Table 1). We observed a positive correlation between the extent of CD4 binding and level of fusogenicity mediated by Env (Fig. 1A). Similar levels of gp120 were expressed on the surface of cells used in CD4-binding assays and cell–cell fusion assays (data not shown). Furthermore, we observed a positive correlation between the extent of CD4 binding and the ability of Env to use low levels of CD4 to mediate fusion (Fig. 1B). The results of CD4 binding, fusogenicity, and relative ability to use low levels of CD4 for the individual Env clones is summarized in Supplementary Table 1. Together, these results suggest that enhanced fusogenicity by primary R5 gp120 proteins is influenced by the ability of gp120 to bind to and utilize CD4.

Structural models of primary R5 gp120 proteins in the CD4-bound conformation and mapping predicted CD4 contact residues

To better understand the molecular mechanisms contributing to alterations in CD4 binding by primary R5 gp120 proteins and the subsequent influence on CD4 dependence and fusogenicity, we produced structural models of CD4-liganded gp120 proteins. The three-dimensional structural similarity between the 2B4C crystal structure of JRFL gp120 and 16 predicted structures of the primary R5 gp120 proteins was <1.0 Å for all the primary gp120 models (range, 0.25 to 0.56 Å) (data not shown), indicating a high overall degree of structural similarity. Identical RMSD values for each gp120 model were obtained upon repeated, independent modeling operations (data not shown). Residues in gp120 where atoms are predicted to be within 4 Å of CD4 were mapped to the molecular surface of all gp120 models, with comparison to the 2B4C crystal structure of JRFL gp120 (Fig. 2). These residues were generally conserved across all primary gp120 proteins and included those identified previously to be involved in CD4 binding (Kwong et al., 1998). Residues at the gp120–CD4 interface were mapped to the base of the V1V2 loop, C2, C3, C4, and V5 regions of gp120. The number of potential CD4 contact residues in gp120, as determined using the Protein Interfaces, Surfaces and Assemblies (PISA) computational platform (Krissinel

Table 1
Coreceptor usage and fusion activity of Env clones.

Env clone	Primary virus isolate ^a	Coreceptor usage ^b	Fusion activity ^b	GenBank accession number
NB2-C1	NB2	CCR5	++	EU308541
NB2-C4		CCR5	+++	EU308543
NB6-C3	NB6	CCR5	++	EU308546
NB6-C4		CCR5	+++	EU308547
NB7-C1	NB7	CCR5	++++	EU308548
NB7-C2		CCR5	+++	EU308549
NB8-C2	NB8	CCR5	+++	EU308553
NB8-C4		CCR5	++	EU308555
NB23-C2	NB23	CCR5	+	EU308557
NB23-C3		CCR5	+	EU308558
NB24-C3	NB24	CCR5	++	EU308561
NB24-C4		CCR5	++	EU308562
NB25-C2	NB25	CCR5	+	EU308564
NB25-C3		CCR5	+	EU308565
NB27-C2	NB27	CCR5	++	EU308567
NB27-C3		CCR5	++	EU308568

^a The primary virus isolates and the clinical characteristics of the subjects from whom they were isolated have been described in detail previously (Gray et al., 2005; Li et al., 1999).

^b The coreceptor usage and fusion activity of the Env clones has been described previously (Sterjovski et al., 2007). Fusion activity was scored as + (50–100 × 10⁵ RLU), ++ (100–200 × 10⁵ RLU), +++ (200–300 × 10⁵ RLU), or ++++ (>300 × 10⁵ RLU).

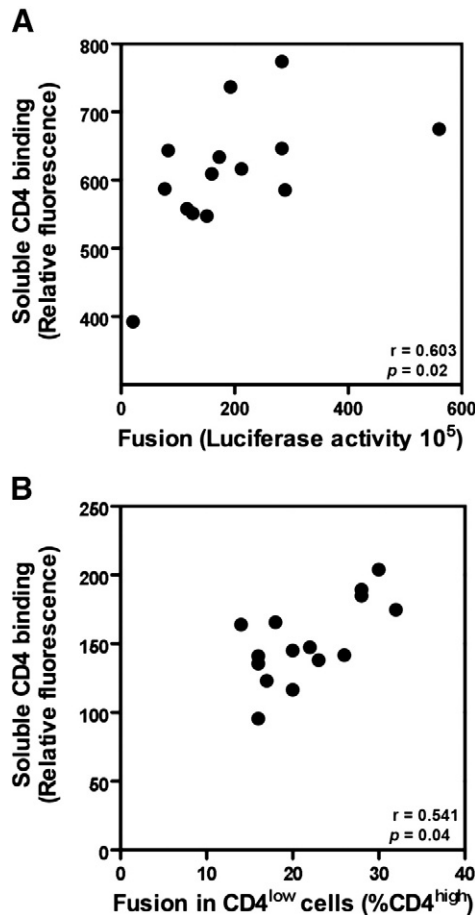


Fig. 1. Association between gp120–CD4 binding, fusogenicity, and CD4 dependence. The ability of gp120 to bind to CD4 was quantified as described in [Materials and methods](#) and plotted against the overall level of fusion mediated by gp120 in cell–cell fusion assays (A), or the relative ability of gp120 to mediate fusion in CD4^{low} cells (B), using Prism version 4.0c (GraphPad Software). The Spearman correlation coefficient (r) and P values are shown. P values <0.05 were considered statistically significant. The data shown are representative of 3 independent experiments.

and Henrick, 2007), was not associated with CD4 binding, fusogenicity or CD4 dependence of the primary R5 gp120 proteins (data not shown), suggesting that the type or position of amino acid in the CD4bs rather than the number of contacts may enhance the CD4-binding properties of gp120.

Five amino acid variants within or immediately adjacent to the CD4-binding regions were identified at positions 195 in the V1V2 region, 279 and 283 in the C2 region, and 362 and 363 in the C3 region of gp120 (Fig. 2C). Modeling these amino acid variants on the CD4-liganded JRFL crystal structure shows that they cluster around the CD4-binding pocket of gp120 with the potential to influence CD4 binding (Fig. 3). Previous mutagenesis studies have shown that amino acid variation at position 283 affects CD4bs exposure and CD4 binding of gp120 proteins derived from brain and other tissues (Duenas-Decamp et al., 2009; Dunfee et al., 2006; Peters et al., 2006). Further mutagenesis studies have shown that variation at position 363 influences CD4 binding (Duenas-Decamp et al., 2009) and that variation at position 362 influences fusogenicity (Sterjovski et al., 2007). Thus, the amino acid polymorphisms identified within or surrounding the gp120 CD4bs may influence gp120–CD4 interactions.

The effect of D279 and N362 on CD4 dependence

To better understand the role of CD4bs polymorphisms on the interaction between gp120 and CD4, the association between the

presence of amino acid variants identified within the CD4bs of primary R5 gp120 models and ability of gp120 to utilize CD4 to mediate fusion was determined. The presence of D279 (Fig. 4A) was associated with a relatively modest yet statistically significant reduction in CD4 dependence (Fig. 4B) when compared to gp120 proteins containing the clade B consensus residue N279. Analysis of potential molecular bond partners showed that, while N279 does not directly contact residues in CD4, the presence of the negatively charged Asp residue at this position has the potential to form an ion pair with the positively charged K31 residue of CD4 (Fig. 4C, D). Thus, an additional salt bridge formed between D279 of gp120 and K31 of CD4 may strengthen the gp120–CD4 interaction. Further mutagenesis studies are required to confirm this hypothesis. Similarly, the presence of N362 (Fig. 5A), shown previously by mutagenesis studies to enhance the fusogenicity of gp120 (Sterjovski et al., 2007), was associated with reduced CD4 dependence in the majority of clones containing this residue (Fig. 5B) compared to gp120 proteins that lacked N362. N362 has the potential to form an additional hydrogen bond with one or more residues within the β 24 and β 28 strands within the CD4-binding pocket of gp120 (Sterjovski et al., 2007), suggesting that N362 may augment the gp120–CD4 interaction by stabilizing the CD4-bound conformation of gp120. Furthermore, N362 is potentially glycosylated, whereby a branched N-linked carbohydrate moiety is likely to protrude along the first and possibly the second domain of CD4 and may further stabilize the gp120–CD4 interaction (Fig. 5C, D). There was no association between CD4bs polymorphisms at positions 195, 283, or 363 and reduced CD4 dependence by the Envs studied here (data not shown). Thus, D279 and N362 CD4bs variants that were identified by homology modeling may contribute to enhanced CD4 interactions of these primary R5 gp120 proteins.

Conformational alterations in the CD4bs influence CD4-binding and Env fusogenicity

The preceding studies identified two CD4bs polymorphisms associated with increased ability of gp120 to utilize CD4. However, these polymorphisms could not fully account for the magnitude of reduced CD4 dependence. Therefore, to determine whether gp120–CD4 interactions are also influenced by changes in the overall conformation of the CD4bs, the aperture width of the CD4bs cavity was calculated from each of the three-dimensional gp120 structural models by measuring the distance between the inner most atoms present at the stem of the V1V2 loops and the V5 loop, which constrain the CD4-binding pocket of gp120 (Huang et al., 2005). Since previous studies from Kwong and colleagues showed that changes in CD4-binding affinity are due to structural alterations involving the variable loop regions rather than from elements within the gp120 core (Kwong et al., 2000), we specifically chose a measurement spanning the base of V1V2 to V5 rather than other distance measurements across the CD4bs cavity. Fig. 6A shows the calculation of the CD4bs aperture width for the NB23–C3 gp120 model as an example, and the results for all the structural models are summarized in Fig. 6B.

We next determined whether this predicted conformational alteration in the CD4bs may potentially affect CD4 interactions and gp120 function. We found a positive correlation between the predicted width of the CD4bs cavity in gp120 and the ability of gp120 to bind CD4 (Fig. 7A), suggesting that a wider CD4bs cavity may augment the gp120–CD4 interaction. Furthermore, we found a near-significant association between the width of the CD4bs cavity and level of fusogenicity mediated by Env (Fig. 7B). Together, these results provide evidence for a conformational alteration in the CD4bs of gp120 that is associated with increased ability of gp120 to interact with CD4 and augmented fusogenicity of Env.

The gp120 V5 loop influences conformational alterations in the CD4bs

We next investigated determinants in gp120, which may contribute to predicted alterations in the width of the CD4bs cavity and which subsequently influence CD4 binding and fusogenicity. Structural overlays of NB25-C3 and NB6-C3 gp120 models, which are gp120 proteins predicted to have the narrowest and widest of the CD4bs apertures, respectively (Fig. 6B), revealed a high degree of structural similarity within the V1V2 stem region, but notable structural variation within the V5 loop region (Fig. 8A). Furthermore, sequence analysis of all the primary R5 Envs showed a high degree sequence homology within the V1V2 stem region (Fig. 8B) but a high degree of sequence variation within the V5 loop (Fig. 8C). Together, these results suggest that alteration in the width of the CD4bs cavity may be due to sequence variability in V5, which may contribute to repositioning of the V5 loop. This potential structural alteration, along with possible effects of CD4bs polymorphisms, may increase the ability of gp120 to interact with CD4 and mediate fusion.

Discussion

In this study, we characterized Env determinants that contribute to enhanced fusogenicity, which is an important pathophysiological phenotype of R5 HIV-1 strains that are present at late stages of HIV-1 infection, and of pathogenic SHIV variants following passage in macaques. We showed that increased fusogenicity of primary R5 HIV-1 Envs is associated with enhanced gp120–CD4 binding and subsequently, an increased ability of Env to use low levels of cell-surface CD4. Therefore, alterations in Env, which augment the ability of gp120 to interact with CD4, influence the fusogenic properties of Env. Consistent with our results, a number of studies have shown that certain highly M-tropic HIV-1 strains, particularly those isolated from the brain or other tissues that favor macrophage replication of virus, have enhanced gp120–CD4-binding capacity and/or greater exposure of the gp120 CD4bs (Duenas-Decamp et al., 2009; Dunfee et al., 2006, 2007, 2009; Gorro et al., 2001, 2002; Martin et al., 2001; Martin-Garcia et al., 2005; Peters et al., 2006, 2008; Rossi et al., 2008; Thomas et al., 2007). This permits engagement with CD4 that is expressed at comparatively low levels on microglia and macrophages. Thus, our results suggest that enhanced fusogenicity of blood-derived R5 Envs most likely results from structural alterations in gp120 that are common to or overlapping with those that promote M-tropism of particular R5 Env variants.

Cross-sectional and longitudinal studies have shown that increased Env-mediated fusogenicity is a prominent phenotype of R5 Envs that contributes to CD4+ T cell decline in subjects who harbor R5 HIV-1 strains to late stages of infection (Sterjovski et al., 2007). Consistent with previous studies that showed membrane fusing capacity to be essential for Env-mediated cytopathicity *in vitro* (LaBonte et al., 2000), particularly in cells where CD4 levels are limiting (Wade et al., 2010), the results of our studies suggest that increased Env-mediated fusogenicity may reflect an increased ability of Envs to cause cytopathic effects through enhanced gp120–CD4 binding. This idea is supported by studies in macaques, where passage of chimeric SHIV strains led to enhancement of pathogenicity associated with adaptive changes in Env (Cayabyab et al., 1999; Karlsson et al., 1997, 1998; Liu et al., 1999; Stephens et al., 1996, 1997). These mutations arose in the gp120 C2, C3, V3, V4, and gp41 Env regions, resulting in increased Env-mediated fusogenicity that was thought to occur via increased Env-receptor binding. Thus, increased fusogenicity contributes to viral pathogenicity in the macaque model. The cytopathic effects of Env-mediated fusogenicity are also evident in humans as MNGC, which are present in autopsy brain tissues of subjects with HIVE (Price, 1996) and formed by Env-mediated fusion between infected and uninfected macrophage-lineage cells (Gonzalez-Scarano and Martin-Garcia, 2005). Multinu-

cleated giant cells in brain are caused predominantly by R5 HIV-1 Envs (Gorro et al., 2001, 2002; Peters et al., 2004; Shieh et al., 1998), which share several features with late-emerging blood-derived R5 viruses such as enhanced fusogenicity (Gorro et al., 2002; Peters et al., 2004; Thomas et al., 2007), increased sensitivity to neutralization by IgG1b12 (Dunfee et al., 2009; Gorro et al., 2002), and Env structures that enable efficient Env–CD4 interactions (Dunfee et al., 2006). Thus, increased fusogenicity of blood-derived R5 Envs may enhance their cytopathic potential via increased gp120–CD4 interactions.

To better understand the nature of gp120 structural alterations that could be involved in augmenting gp120–CD4 interactions, we produced 3-dimensional structural models of gp120 sequences, using the CD4-bound 2B4C gp120 crystal structure as template. While acknowledging the limitations of gp120 models for accurately interpreting structural alterations (discussed in more detail below), we carefully validated our approach by first comparing the JRFL crystal structure to the predicted structure of the same gp120 sequence after extensive optimization of the modeling protocol. The RMSD between the predicted and crystal structures of JRFL gp120 after rigid-body superposition of C α atoms of the entire gp120 structure was 0.37 Å, indicating a high degree of structural similarity, providing evidence that the gp120 models used in this study have sufficient accuracy to provide useful structural information. Thus, cautious interpretation of structural models of gp120 based on our optimised protocol may shed light on potential gp120 alterations, which may contribute to enhanced CD4 interactions and fusogenicity.

Analysis of predicted three-dimensional gp120 structural models revealed a potential CD4bs alteration that appears to be involved in modulating the ability of gp120 to interact with CD4. Specifically, a wider aperture of the predicted CD4bs cavity, as constrained by the inner-most atoms at the gp120 V1V2 stem and the V5 loop, was associated with increased gp120–CD4 binding and increased fusogenicity. In contrast, we did not observe significant trends between the overall area of the three-dimensional CD4bs cavity, as determined by analysis using the PISA computational platform (Krissinel and Henrick, 2007), and CD4 binding or fusogenicity (data not shown), suggesting that a specific CD4bs conformation rather than changes in the total contact area of the CD4bs may influence interactions between gp120 and CD4. Thus, our results provide evidence for structural alterations within the gp120 CD4bs that influence Env-mediated fusogenicity through an increased ability to interact with CD4. In addition to studying mechanisms of HIV-1 fusogenicity, the CD4bs modeling approach described here may be useful for further elucidating the molecular determinants of HIV-1 neurotropism, which are known to involve alterations in gp120–CD4 binding.

CD4 binding to gp120 results in conformational changes thought to be the result of an “induced fit” to the CD4 molecule (Kwong et al., 1998). This suggests that greater CD4 affinity should result in a tighter interaction between gp120 and CD4, rather than a more open conformation. However, since CCR5 is more mobile on the surface of cells than CD4 (Steffens and Hope, 2004), a more open conformation of the CD4bs cavity may instead allow greater flexibility that minimizes steric constraints during the process of CCR5 sequestration. This suggests that a steric advantage, rather than or in addition to increased affinity for CD4, may drive the selection of primary R5 Envs with a wider CD4-binding site cavity. The structural rearrangement of gp120 that occurs upon CD4-binding results in residues that would otherwise be exposed on the surface of gp120 to become buried at either the CD4 interface or at the junctions of gp120 domains that are drawn together by CD4 (Myszka et al., 2000). Analysis of unbound models of primary gp120 sequences may identify features in the pre-triggered, unbound state of gp120 that may explain the significance of the movement of the V5 loop in gp120 proteins and other features that could influence the initial contact with CD4.

While our results suggest a possible functional role for CD4bs alterations in the augmented function of certain R5 gp120 proteins,

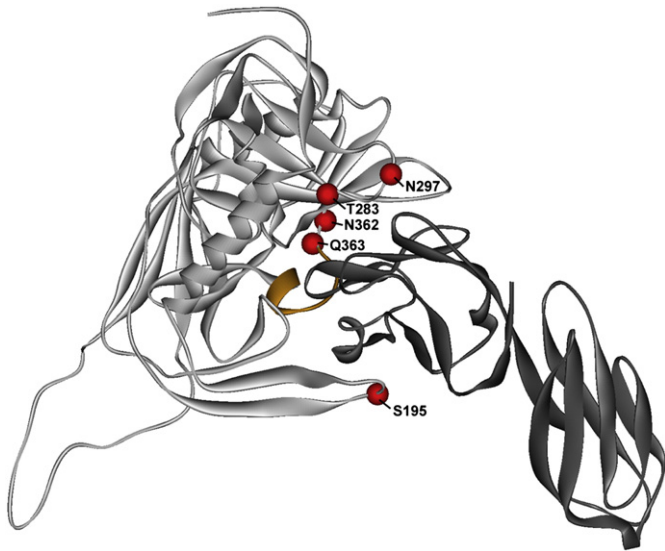


Fig. 3. CD4bs variants modeled on the crystal structure of HIV-1 JRFL gp120. JRFL gp120 is shown in ribbon representation (light grey) in complex with CD4 (dark grey). The C α atoms of amino acid variants N297, T283, N362, Q363, and S195 are displayed as space filling spheres (red). The CD4-binding loop of gp120 is shown as orange ribbon.

there are a number of important considerations to take into account when predicting gp120 structures by homology modeling. The first is whether homology modeling can reliably predict the structure of protein loops, such as V5. Loop building strategies are well accepted to be accurate for predicting the structure of relatively small protein loops like V5 (Eswar et al., 2007). Thus, while these algorithms are likely to have limitations for predicting the structures of larger protein loops such as the gp120 V3 loop, they are likely to be accurate for predicting V5 loop structures. Second, the V1V2 gp120 loops are missing from the 2B4C JRFL crystal structure, and therefore, these regions are not accounted for in the gp120 models. The V1V2 loops have been previously shown to modulate immunogenicity and conformational shielding of the CD4 and coreceptor binding sites (Bouma et al., 2003; Ching et al., 2008; Fox et al., 1997; Ly and Stamatatos, 2000; Nabatov et al., 2004; Pinter et al., 2004) and, therefore, will likely exert an affect on the structure of the CD4bs. Finally, the gp120 models represent predicted monomeric proteins, which are unlikely to completely recapitulate the functional properties of trimeric gp120. Therefore, a fully glycosylated trimeric gp120 structure in the native, unbound form that includes all the variable loop regions is required in order to fully understand the influence of CD4bs alterations on the function of primary R5 gp120 proteins. Nonetheless, in the absence of such a structure, the cautious

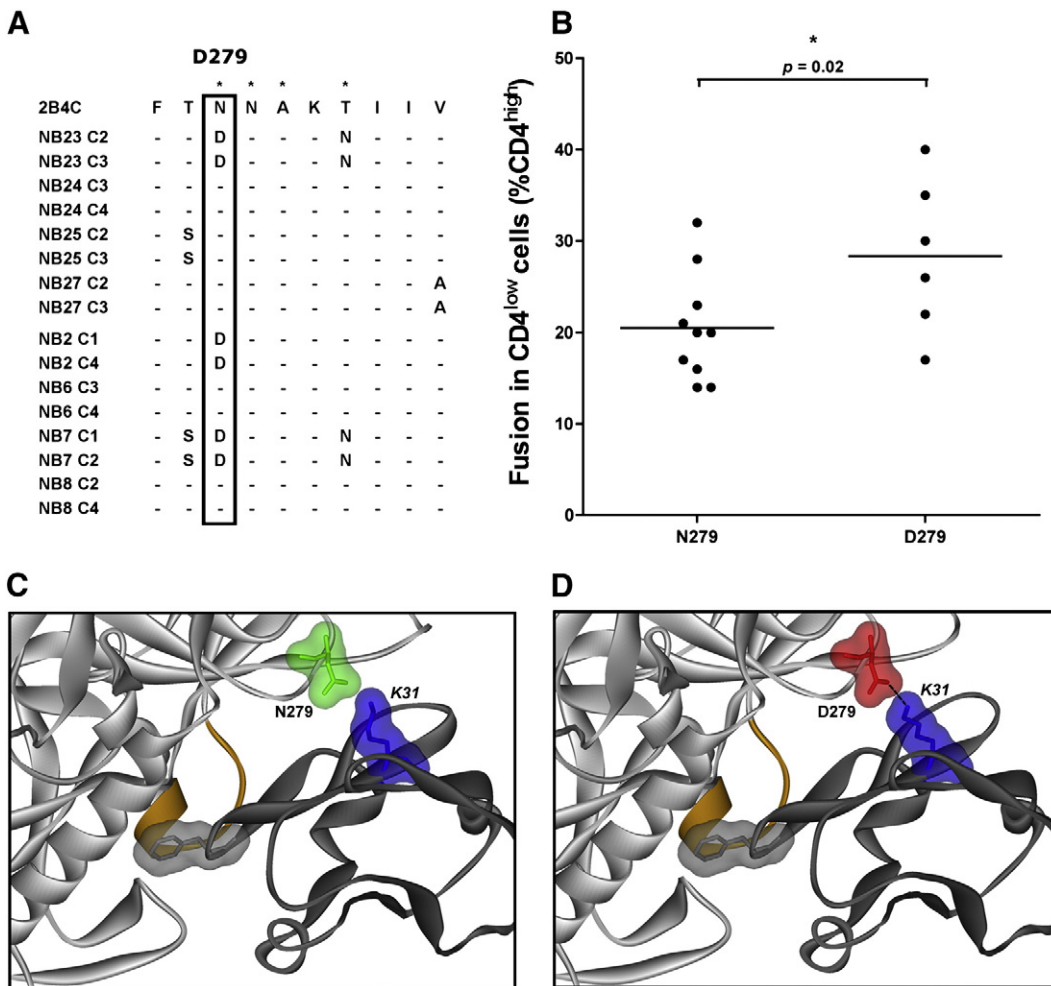


Fig. 4. The effect of D279 on gp120–CD4 interactions. (A) Sequence alignment illustrating the D279 polymorphism located within the C2 region of gp120. (B) For all Env clones, the relative ability of gp120 to mediate fusion in CD4^{low} cells was measured as described in Materials and methods and stratified based on the presence of N279 or D279. The data shown are representative of 3 independent experiments. *P* values were calculated using a nonparametric Mann–Whitney *U* test, and values <0.05 were considered statistically significant. N279 was modeled on NB24–C3 gp120 and shown in green stick representation, with van der Waals surface also in green (C). D279 was modeled on NB7–C1 gp120 and shown in red stick representation, with van der Waals surface also red (D). K31 of CD4 is shown in blue stick representation and blue van der Waals surface. Gp120 and CD4 molecules are shown in ribbon representation (light grey and dark grey, respectively), and Phe43 of CD4 is shown in grey stick representation with grey van der Waals surface.

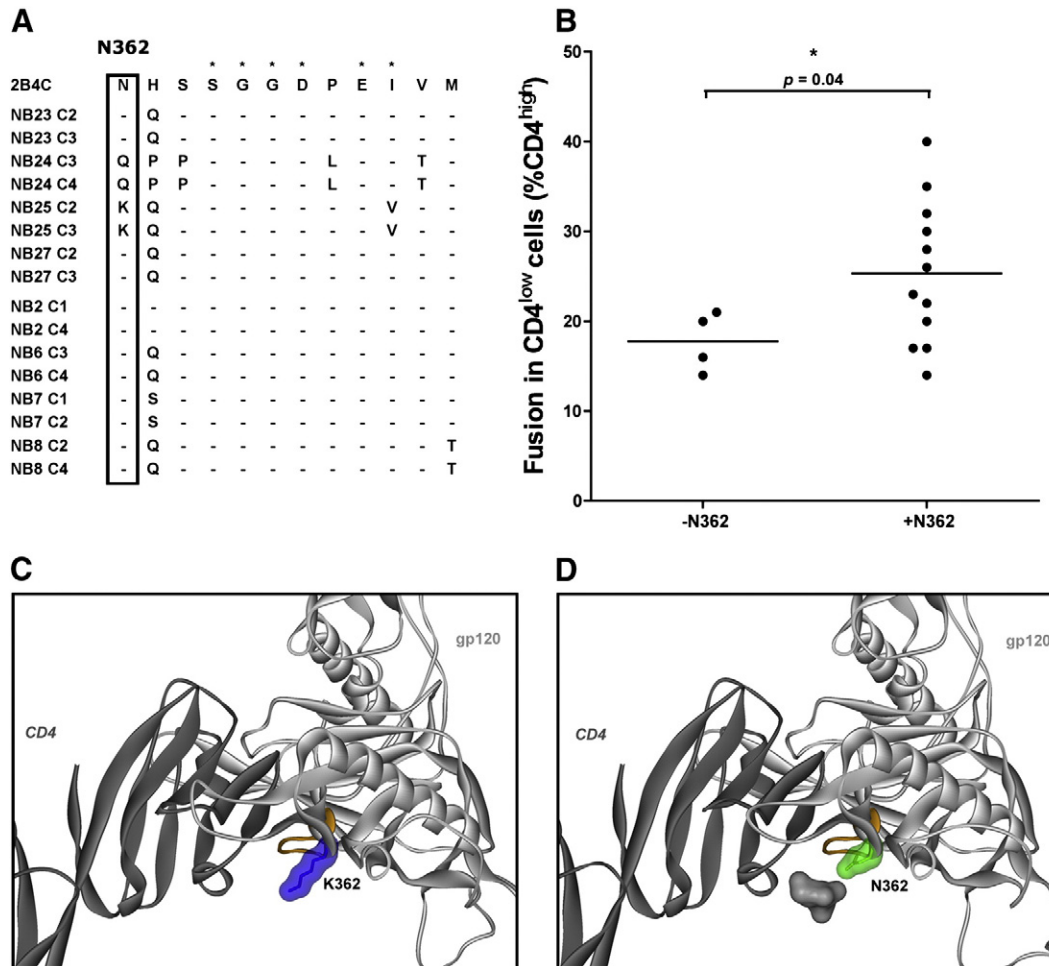


Fig. 5. The effect of N362 on gp120–CD4 interactions. (A) Sequence alignment illustrating the N362 polymorphism located within the C3 region of gp120. (B) For all Env clones, the relative ability of gp120 to mediate fusion in CD4^{low} cells was measured as described in *Materials and methods* and stratified based on the presence or absence of N362. The data shown are representative of 3 independent experiments. *P* values were calculated using a nonparametric Mann–Whitney *U* test, and values <0.05 were considered statistically significant. K362 was modeled on NB25–C2 gp120 and shown in blue stick representation with van der Waals surface also in blue (C). N362 was modeled on NB7–C1 gp120 and shown in green stick representation with van der Waals surface also in green, the potential glycan of N362 is depicted as grey molecular surface (D). Gp120 and CD4 molecules are shown in ribbon representation (light grey and dark grey, respectively).

interpretation of our results based on the 2B4C JRFL crystal structure provides mechanistic insights that are likely to be functionally important.

In summary, our results suggest that alterations in the gp120 V5 loop may influence conformational variation in the gp120 CD4bs. We highlight evidence that an increase in the aperture of the CD4bs

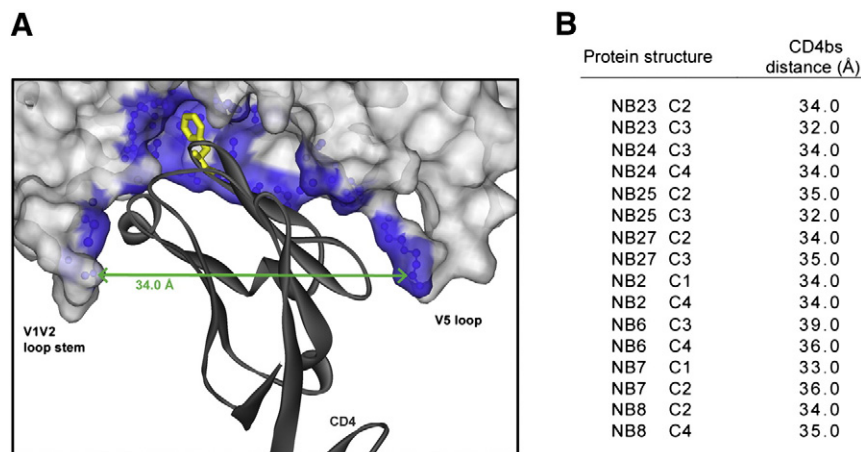


Fig. 6. Predicted alterations in the CD4bs cavity from 3-dimensional gp120 models. (A) The gp120 model of NB23–C2 Env is shown in molecular surface representation, and the CD4 molecule, in grey ribbon, with Phe43 of CD4 in yellow stick representation to highlight the “Phe43 cavity” of gp120. Residues of gp120 within the CD4-binding pocket and located within 4 Å of CD4 are shown in ball and stick representation, and their molecular surface is highlighted in blue. The width of the CD4bs aperture was determined by measuring the distance across the two innermost atoms located at the V1V2 stem and the V5 loop. (B) Summary of the CD4bs aperture widths calculated for all the primary gp120 models.

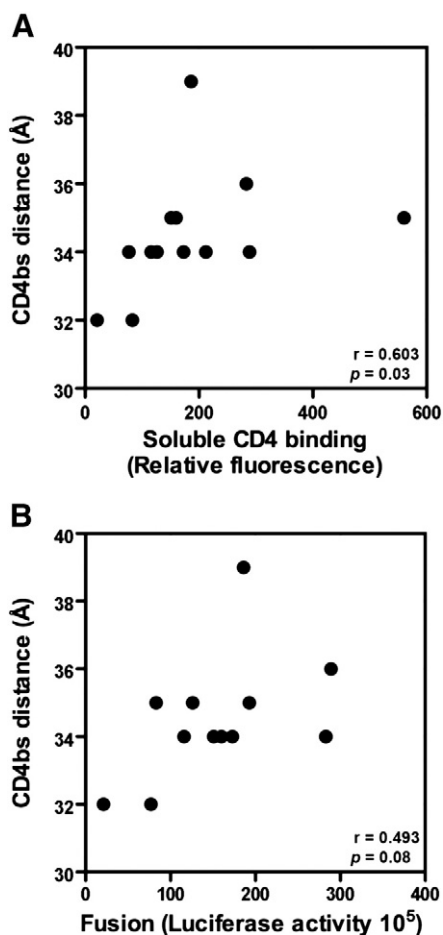


Fig. 7. Association between gp120 CD4bs cavity alterations, CD4 binding, and fusogenicity. The CD4bs aperture widths shown in Fig. 6 were plotted against the ability of gp120 to bind CD4 (A) or mediate cell–cell fusion (B), which were determined as described in Materials and methods, using Prism version 4.0c (GraphPad Software). The Spearman correlation coefficient (r) and P values are shown. P values <0.05 were considered statistically significant. The data shown are representative of 3 independent experiments.

cavity, as constrained by the inner-most atoms at the gp120 V1V2 stem and the V5 loop, influences the ability of gp120 to bind to CD4 and mediate cell–cell fusion. This potential conformational alteration may be an important structural adaptation in HIV-1 contributing to augmented Env function.

Materials and methods

HIV-1 Env clones

The HIV-1 Envs used in this study were cloned from primary R5 HIV-1 isolates, which have been described in detail previously, including the clinical characteristics of the subjects from whom they were isolated (Gray et al., 2005; Li et al., 1999; Sterjovski et al., 2007). The Envs used were NB23-C2, NB23-C3, NB24-C3, NB24-C4, NB25-C2, NB25-C3, NB27-C2, NB27-C3, NB2-C1, NB2-C4, NB6-C3, NB6-C4, NB7-C1, NB7-C2, NB8-C2, and NB8-C4, which are cloned into the pSVIII-Env expression vector (Gao et al., 1996). These clones have been described in detail previously (Sterjovski et al., 2007; Sterjovski et al., 2010), and their coreceptor usage and fusion activities are summarized in Table 1.

Cells

Cf2-Luc cells (Etemad-Moghadam et al., 2000), derived from the Cf2th canine thymocyte cell line (Choe et al., 1996), stably express the

luciferase gene under the control of the HIV-1 long terminal repeat and were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum (FCS), 100 μ g of penicillin and streptomycin per milliliter, and 0.7 mg of G418 per milliliter. 293 T cells were cultured in DMEM supplemented with 10% (vol/vol) FCS and 100 μ g of penicillin and streptomycin per milliliter.

Fusion assays

Cell–cell fusion assays were conducted as described previously (Sterjovski et al., 2007). Briefly, Cf2-Luc target cells seeded in 25 cm² tissue culture flasks were transfected with 1 μ g of CD4 plasmid and 3 μ g of CCR5 plasmid. 293 T effector cells seeded in 6-well tissue culture plates were co-transfected with 3.4 μ g of Env-expressing plasmid and 0.6 μ g pSVL-Tat. Target and effector cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturers' protocol. Approximately 2.5×10^4 293 T effector cells were added to 2.5×10^4 Cf2-Luc target cells. After mixing, target and effector cells were incubated at 37 °C in replicate wells containing 200 μ l of culture medium. Cells from replicate wells were harvested at 10 h post-mixing and assayed for luciferase activity (Promega) according to the manufacturers' protocol. Luminescence was measured using a FLUOStar microplate reader (BMG LABTECH, GmbH, Germany). 293 T cells transfected with pSVL-Tat alone were used as negative controls to determine the background level of luciferase activity. For measurement of CD4 dependence, Cf2-Luc target cells were transfected with 2.5 μ g of CCR5 plasmid together with either 0.5 or 2.5 μ g of CD4 plasmid to generate CD4^{low} and CD4^{high} cells, respectively. This approach results in an increase in the percentage of cells expressing CD4 as well as the levels of cell-surface CD4 expression (data not shown), as we and others have shown previously (Dunfee et al., 2006; Gorry et al., 2001, 2002; Gray et al., 2005; Martin et al., 2001; Martin-Garcia et al., 2005). To measure CD4 dependence, the ability of gp120 to mediate fusion in CD4^{low} cells was expressed as a percentage of the ability to mediate fusion in CD4^{high} cells.

CD4-binding assays

293 T cells were transfected with Env plasmids as described above. At 48 h post-transfection, approximately 2×10^5 cells were used in CD4-binding reactions, using soluble CD4 (sCD4; extracellular domains D1–D4) (Progenics Pharmaceuticals, Tarrytown, NY) that was conjugated to the Atto488 fluorophore (Reamatrix, San Carlos, CA). The levels of Env expressed on the surface of 293 T cells were monitored by flow cytometry, as described previously (Sterjovski et al., 2007). After pre-incubation with FACS wash buffer (FWB) [PBS containing 3.5% (wt/vol) bovine serum albumin, 0.05% (wt/vol) sodium azide, pH 7.3–7.4] for 2 h at RT, cells were washed three times in FWB and resuspended in 20 μ l of FWB containing 2 μ g of sCD4-Atto488. This concentration of sCD4-Atto488 was empirically determined to be within the linear range of Env–CD4 binding and was readily able to discriminate between Envs known to have high CD4-binding capability (e.g., YU-2) versus Envs that bind CD4 more weakly (e.g., JR-CSF) (data not shown). Cells were incubated with sCD4-Atto488 for 1 h at RT, after which they were washed three times with FWB. Cells were then resuspended in 150 μ l of PBS containing 4% (wt/vol) paraformaldehyde and analyzed by flow cytometry as described previously (Gorry et al., 1999).

Computer-aided structure prediction of gp120

Homology models of CD4-bound gp120 sequences were prepared using the Build Model protocol of the Discovery Studio suite, version 1.6 (Accelrys, San Diego, CA, USA), as we have described recently (Sterjovski et al., 2010). This approach used the Modeller algorithm to generate an atomic model of the target protein from a template

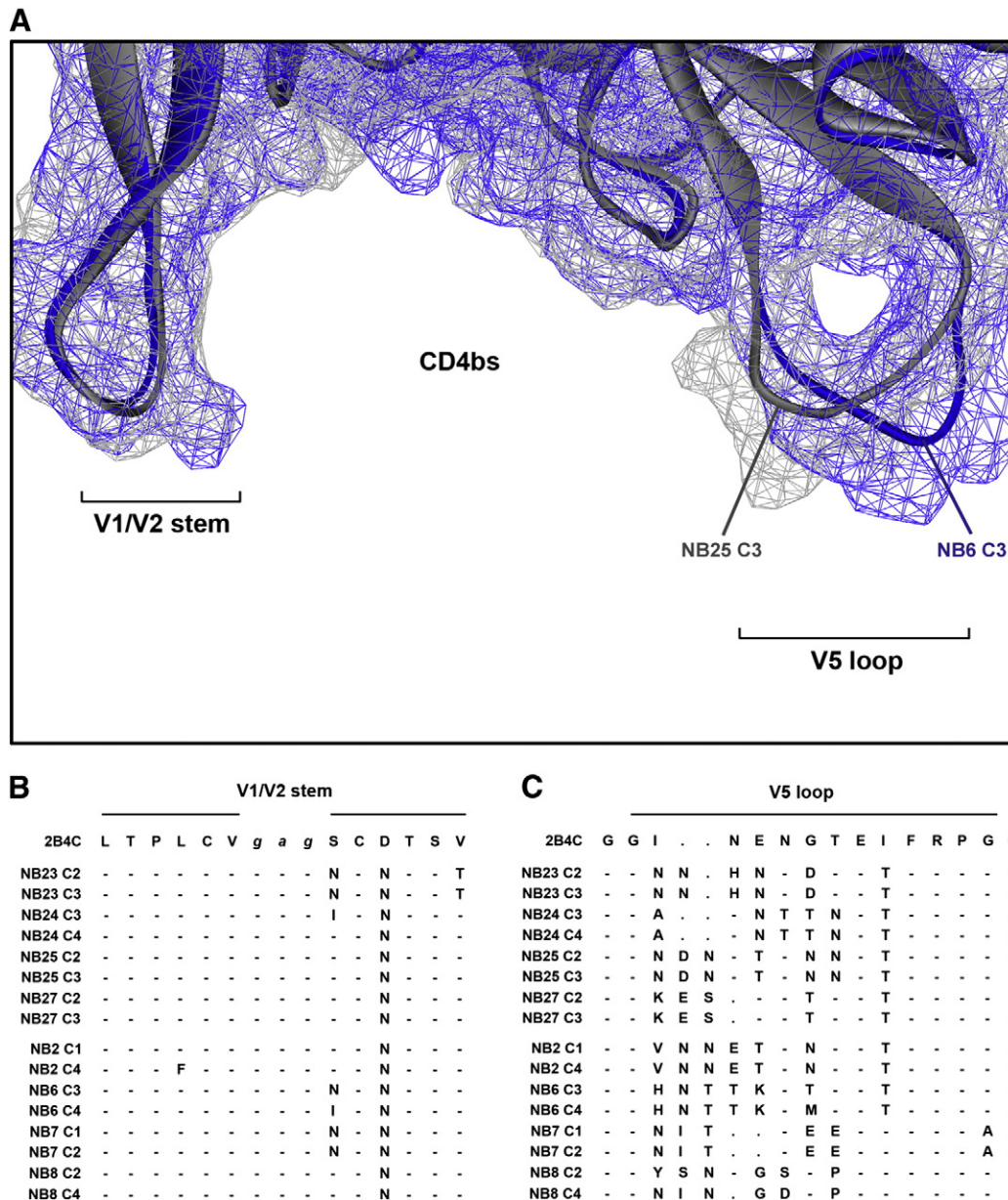


Fig. 8. Repositioning of the V5 loop may contribute to structural alterations in the gp120 CD4bs cavity. (A) The gp120 models of NB25-C3 and NB6-C3 Envs (grey and blue ribbon representation, respectively) were superimposed, and their molecular surfaces were presented as blue or grey wire mesh. Sequences of the V1V2 stem (B) and the V5 loop (C) of the primary gp120 models were aligned against the 2B4C JRFL crystal structure sequence. The GAG linker sequence, which replaced the V1V2 loops in the crystal and model structures, is shown.

molecule and a sequence alignment. The template-based models were optimised by iterative cycles of conjugate-gradient minimization against a probability density function that included spatial restraints derived from the template and residue specific properties (Sali and Blundell, 1993). The crystal structure of JRFL gp120 containing the V3 variable loop and bound to CD4 and the X5 Fab antibody fragment was used as the template for CD4-bound models (Huang et al., 2005) (Protein Data Bank ID: 2B4C). The X5 antibody fragment was deleted from the CD4-bound template prior to modeling. The coordinates for gp120 and CD4 were extracted from the 2B4C crystal structure. Sequence alignments were generated between JRFL gp120 and the primary gp120 Env clones. The sequence for CD4 was included as a second polypeptide chain such that the models of gp120 were constructed as complexes with CD4. The V1/V2 variable loops were replaced with a GAG linker sequence and the N- and C-terminal overhangs were cut using the modeling software.

Similarities in three-dimensional structure were measured by the root mean square deviation (RMSD) of the distances between main-chain atoms (N, C α , C, and O atoms) from crystal and model structures after rigid body superposition, where an RMSD of <1 Å signifies a high level of homology of three-dimensional structure between overlaid proteins. The overall quality of the geometry of gp120 models generated was verified using PROCHECK (Laskowski et al., 1993).

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